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Regulation of the AcrAB-TolC efflux pump in Enterobacteriaceae

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Abstract

Bacterial multidrug efflux systems are a major mechanism of antimicrobial resistance and are fundamental to the physiology of Gram-negative bacteria. The Resistance-Nodulation-Division (RND) family of efflux pumps are the most clinically significant as they are associated with multi-drug resistance. Expression of efflux systems is subject to multiple levels of regulation, involving local and global transcriptional regulation as well as post-transcriptional and post-translational regulation. The best-characterised RND system is AcrAB-TolC, which is present in Enterobacteriaceae. This review describes the current knowledge and new data about the regulation of the *acrAB* and *tolC genes* in *Escherichia coli* and *Salmonella enterica*.

Keywords: AcrAB-TolC, Transcription, Induction, Multidrug resistance, *Salmonella, Escherichia coli*,

Introduction

Bacterial multidrug efflux systems are a major and common mechanism of intrinsic antimicrobial resistance employed by bacteria. Efflux systems are able to extrude a variety of structurally diverse antimicrobials and some metabolites [10]. Many efflux pumps are fundamental to the physiology of some species of Gram-negative bacteria, and are required for virulence and formation of biofilms [10]. The most clinically significant efflux pumps in Gram-negative bacteria are members of the Resistance-Nodulation-Division (RND) family as they recognise a broad range of substrates and are associated with multi-drug resistance (MDR) [10]. These pumps exist as a tripartite system, spanning both the inner and outer membrane [10, 5] . The best characterised RND system is AcrAB-TolC, and is present in Enterobacteriaceae including *Escherichia coli, Salmonella* species and *Klebsiella pneumoniae*. Pumps homologous to AcrAB-TolC exist in other species of Gram-negative bacteria, such as MexAB-OprM, MexCD-OprJ and MexXY-OprM in *Pseudomonas spp.*, CmeABC in *Campylobacter spp*., MtrCDE in *Neisseria spp*. and AdeABC in *Acinetobacter baumannii* [19, 25, 26, 28, 30, 36, 48, 27].

Expression of efflux systems is subject to multiple levels of regulation, involving local and global transcriptional regulation as well as post-transcriptional and post-translational regulation. As RND MDR efflux systems show high levels of homology at the gene and protein level, AcrAB-TolC has been studied most extensively as the prototype of this class of pumps [27] . In *E. coli,* expression of the *acrAB* and *tolC* genes is primarily controlled by MarA, whereas in *Salmonella enterica* these genes are controlled by RamA. This review will focus on the current state of knowledge of the regulation of the *acrAB* and *tolC genes* in *E. coli* and *S.*

enterica, respectively. We also describe other factors that have been recently discovered.

Local transcription regulation

The mechanism of RND MDR efflux pump regulation is broadly similar in different species; there is local repression of pump genes as well as global transcription factor regulation. In *E coli, Salmonella spp.* and *Klebsiella spp.*, the local repressor AcrR acts as a modulator to prevent the over-expression of *acrAB* [29, 47, 72]. AcrR has been extensively studied in *E. coli*. It is part of the TetR family of transcriptional repressors and when induced it represses *acrAB* [64]. *acrR* is located upstream of the *acrAB* operon, is transcribed divergently and can repress its' own synthesis(Fig.1) [29]. Clinical and veterinary isolates of *E. coli* and *Salmonella* have been identified with mutations in *acrR* that lead to loss of repression and subsequent over expression of AcrAB-TolC and MDR [47, 72]. Transcription of *acrR* is increased under general stress conditions, including 4% ethanol, 0.5M NaCl and the onset of stationary phase in Luria-Bertani (LB) medium [29]. In the absence of functional AcrR, these stress conditions were shown to induce *acrAB*, indicating that AcrR does not act in isolation [29, 72].

Other proteins that locally regulate *acrAB* and *tolC* include AcrS/EnvR, the histone-like nucleoid structuring protein (H-NS), and Suppressor of Division Inhibition (Sdi)A [27]. AcrS/EnvR is a repressor of the *acrEF* efflux pump genes; it can also repress *acrAB* in *E. coli* [21]. AcrS/EnvR may repress *acrAB* in response to increased activity in *acrEF*, allowing cross-regulation of RND efflux pumps [21]. In *S. enterica* and *E. coli*, H-NS regulates expression of genes by responding to environmental signals such as pH, osmolarity and temperature; it also down regulates expression of *acrEF* but not *acrAB* [14, 43, 44]. SdiA, a LuxR protein

present in *E. coli and S. enterica,* can positively regulate *acrAB* [42, 49]. These regulators are thought to play a minor role in regulation of *acrAB* and *tolC* as deletion of these genes results in little effect in efflux via AcrAB [27].

Mar regulation of *acrAB-tolC* in *E. coli*

The multiple antibiotic resistance operon, Mar, in *E. coli* was discovered by a transposon insertion in *marA.* It is expressed as two separate transcriptional units controlled by a common region of DNA, *marO* [13]. *marR, marA* and *marB* genes are involved in multiple antibiotic resistance, however, the *marC* gene, a putative transmembrane protein, is not [37].

MarR is a protein that blocks its own transcription in the absence of any environmental signal [4]. The MarR family of proteins have a unique ability to sense phenolic compounds. MarR recognises and binds palindromic DNA sequences as dimers and the DNA binding domain contains a helix-turn-helix (HTH) motif that favours this activity [3, 62, 65]. Under normal conditions, MarR represses the *marRAB* operon by binding to two palindromic sequences within the operator DNA sequence *marO* that contains its promoter [33]. Transcription of *marRAB* will only occur when repression by MarR is disrupted. This can be due to the presence of certain ligands (e.g. phenolic compounds such as sodium salicylate), antibiotics, oxidative stress or mutation of *marR*, *marO* or the MarR binding site [12].

De-repression of the Mar operon leads to expression of *marA* that encodes a global transcriptional activator, MarA, a member of the AraC/XylS family of proteins. This family has a unique feature: a 100 amino acid sequence that forms a domain that contains two helix-turn-helix (HTH) motifs that bind DNA [35]. MarR always represses *marA*. MarA undergoes positive feedback as it binds to a DNA sequence

upstream of the repression site of MarR known as the marbox. This represses *marR* allowing *marA* to be activated (Fig. 1). The marbox is usually 20 bp in length, highly degenerate and asymmetric [32]. There are approximately 10,000 copies of the marbox in the *E. coli* chromosome but most are inactive [32]. Structural studies show that MarA utilises the two HTH motifs containing the recognition helices 3 and 6 that bind two major grooves on the DNA as a monomer and bends the DNA by 35° (Fig. 2) [16, 50]. Expression of MarA leads to activation of many genes in its regulon including *acrAB* and *tolC* giving increased drug efflux and multiple drug resistance[46]. In the absence of any environment signal or mutation, MarR binds the operator sites and repression of the Mar operon resumes (Fig. 1).

MarB is transcribed as a part of the second transcription unit of the *mar* locus. *marB* is located downstream of *marA* and has its own ribosome binding site [13]. MarB increases the level of MarA by an unknown mechanism, however, its' predicted periplasmic signal sequence suggests that MarB can act post-transcriptionally [68].

The complete *mar* regulon (also known as *mar/sox/rob* regulon) is poorly defined. This is partly because the MarA homologues, SoxS and Rob, which also belong to the AraC family, recognise the same DNA sequence as MarA and regulate transcription similarly [14, 35]. Transcription of *marRAB* and *acrAB-tolC* can also be driven by factors other than MarR and MarA. The MarA homologues SoxS and Rob also bind the same DNA sequence as MarA and are known to activate transcription of *marRAB* and *acrAB-tolC* [39, 40]. The activation of *marRAB* increases when another factor known as Factor for Inversion Stimulation (FIS) binds upstream of the marbox. This activation was only seen in the presence of MarA, RobandSoxS [34]. The expression of MarA, SoxS and Rob is influenced by specific environmental

stimuli, but together ensure appropriate efflux pump regulation via a variety of stress signals.

Like MarA, SoxS is a small protein, of 107 amino acids, formed of a single domain containing two HTH motifs connected by a 27 Å rigid helix [31, 32]. SoxS proteins share 41% and 67% identity and similarity with MarA respectively[35]. Unlike most AraC family proteins, MarA and SoxS have no ligand sensing or dimerisation domain. In the absence of any stress signals the repressor SoxR binds to the *soxS* promoter and represses the transcription of SoxS [20]. Oxidative stress activates *soxS* and SoxS can repress its own expression. MarA and Rob have also been shown to repress the level of SoxS [11, 45].

Rob is a 289 amino acid protein [35]. It is formed of two domains, of which the N-terminal domain shares 51% and 71% identity and similarity with MarA [2, 35]. The N- terminal domain has been shown to have two HTH motifs similar to MarA. Both MarA HTH motifs interact with the DNA major groove, but Rob interacts with DNA major groove utilizing only one HTH motif [23]. The other motif is responsible for binding to the DNA backbone and so the DNA remains unbent [23]. Rob differs from MarA and SoxS by virtue of its second domain; a C-terminal domain that may be involved in ligand binding [23]. Similar to MarA and SoxS, Rob is constitutively expressed and is abundant. Rob binds with higher affinity than MarA or SoxS to the marbox, however, most of the Rob proteins are inactive due to post-transcriptional sequestration [17]. This prevents activation and in turn prevents activation of other promoters regulated by Rob [17]. Sequestrated Rob clumps are not formed when compounds such as 2,2′-dipyridyl activate Rob [60]. When activated, Rob regulates many promoters by binding to the marbox in response to antibiotics and organic solvents [22]. MarA, SoxS and Rob can repress the transcription of Rob [11, 38, 61].

Over-expression of these transcription factors has been found in antibiotic resistant veterinary and human isolates of *E. coli.* Antibiotic resistance in these isolates is often multi-factorial with resistant isolates harbouring multiple mutations, however, the most resistant isolates typically show increased efflux [15]. This is not caused by consistent over-expression of a single transcription regulator, but rather a combination where some isolates show over-expression of MarA and/or SoxS [71]. This correlates with laboratory mutants (Table 1) and re-enforces the evidence that in *E. coli* several global regulators control production of AcrAB-TolC.

Until recently, it was thought that *mar* mutations caused MDR solely by over-expressing the AcrAB-TolC efflux pump, however, the *mar* system regulates other genes as well as *acrAB* and some are involved in resistance to certain classes of drugs. By carrying out ChIP-Seq experiments in ETEC H10407 (which carries a MarR mutation), Sharma *et al*., [63], identified 33 target genes that are regulated by MarA. In addition to the *acrAB* and*tolC* genes, MarA targets genes that play a role in transport, DNA damage repair, and transcription regulation. These include *xseA* which when deleted conferred increased susceptibility to ciprofloxacin, and *mlaFEDCB* which when deleted conferred increased susceptibility to tetracycline [63]. The authors inferred that with AcrAB-TolC, MarA mediates drug resistance.

Ram regulation in *Salmonella*

RamA is an AraC/XylS transcription activator that is a homologue of MarA and regulates expression of the genes encoding the AcrAB-TolC MDR efflux pump [1]. It has a similar structure to MarA and binds to DNA via a similar mechanism [53]. Like MarA in *E. coli*, RamA regulates expression of *acrAB* and *tolC* in *S. enterica* [55] and other Enterobacteriaceae, including *Klebsiella pneumoniae, Enterobacter aerogenes*

and *Enterobacter cloacae* [55]. MarA, SoxS and Rob are also found in these species and they too play a role in expression of *acrAB*. However, RamA functions as the primary regulator [42]. RamA is not found in *E. coli* or *Shigella species* [55].

Like MarA, RamA activates *acrAB* and *tolC* by directly binding to a degenerate nucleotide sequence upstream of the *acrAB* and *tolC* loci known as the rambox [42]. When RamA is constitutively expressed at high levels bacteria are MDR [56]. When *ramA* is highly expressed, there is a concomitant over-expression of *acrAB*, and when *ramA* is inactivated the expression of *acrAB* is reduced [6]. Overproduction of AcrB and increased expression of the AcrAB-TolC efflux pump confers MDR [6]. When *acrB* is inactivated, there is a fourfold increase in levels of *ramA* suggesting a feedback mechanism [6]. In the absence of *ramA* it is also difficult to select MDR mutants suggesting that *ramA* is required for MDR [57].

The *ramR* gene is located upstream of *ramA*; RamR is a repressor of *ramA* transcription (Fig. 1). Point mutations and insertions in *ramR* and the region between *ramA* and *ramR* have been identified in MDR clinical and veterinary isolates of *S.* Typhimurium and other *S. enterica* serovars[1, 55]. These mutations and insertions prevent RamR from binding to the *ramA* promoter causing increased expression of RamA and subsequent increase in expression of AcrAB-TolC and MDR [1, 54, 55, 75]. The RamR binding site is 28 bp in length and covers the essential features of the promoter region of *ramA*, including the -10 conserved region, the transcriptional start site of *ramA* and two 7 bp inverted repeats[8]. By determining the crystal structure of RamR Yamasaki *et al*., identified substrates of the RamR protein which include crystal violet, ethidium bromide and rhodamine 6G [74]. All the compounds

tested were found to interact with various amino acid residues of RamR, reduce DNA-binding affinity, and induce over-expression of *ramA* [74].

Expression of *ramA* is under multilevel control, with several factors stimulating expression of this transcription factor. RamA is synthesized *de novo* in response to inducers [1]. Various environmental stimuli have been shown to influence efflux by AcrAB by increasing expression of *ramA*. Baucheron *et al* demonstrated that bile activates *acrAB* through de-repression of *ramA* [9]. Nikaido *et al* demonstrated increased expression of *ramA* in response to the bacterial metabolite indole as it enhanced the promotor activity of *ramA* [42]. Bailey *et al* demonstrated that phenothiazides and serotonin-uptake inhibitors such as amitriptyline, induced *ramA* expression and were associated with a phenotype of efflux inhibition [6]. Chlorpromazine induced the expression of *ramA* in wild-type *Salmonella* and there was a concomitant decrease in the level of *acrB* transcript [6]. Lawler *et al* further demonstrated that exposure of *Salmonella* to several biocides, and certain antibiotics such as chloramphenicol, ciprofloxacin, rifampicin, cloxacillin and cefamandole also increased *ramA* expression [24].

Interestingly, not all antibiotics which are exported via the AcrAB-TolC efflux pump directly increase *ramA* expression, and over-expression of *ramA* is greatest when part of the AcrAB-TolC pump is inactivated leading to lack of the tripartite pump [24]. Seventeen antibiotics known to be exported via the AcrAB-TolC efflux pump were tested by Lawler *et al*, and only five significantly increased expression of *ramA* [24]. This may suggest, that *ramA* expression is not always triggered by direct action of specific chemical compounds to RamR, but also that the up-regulation of *ramA* may occur in response to other stimuli [24].

Environmental factors involved in regulation of *acrAB-tolC*

The induction of *acrAB-tolC* in the presence of environmental factors such as bile, fatty acids or cationic peptides, is clinically relevant due to Enterobacteraciae encountering these compounds inside the gastro intestinal tract of the host. In *E. coli* binding of bile, fatty acids and cationic peptides to Rob produces conformational changes and so it induces *acrAB* [58, 70]. In *Salmonella spp.* induction of *acrAB* by bile is dependent on RamA; this occurs by bile inhibiting the binding of RamR to the promoter region of *ramA* [9]. Induction of *acrAB*, in both *E. coli* and *Salmonella spp*., can also occur in situations where the cell is under oxidative stress. This is SoxRS dependent and requires the oxidation of the iron–sulphur clusters in SoxR, which in turn induces production of SoxS resulting in the induction of *acrAB* [73]. Induction of *acrAB* can also be triggered by salicylate, a phenolic phytohormone implicated in plant defence against bacterial pathogens. Salicylate binds to MarR, causing conformational changes which leads to disassociation of MarR from the *marRAB* promoter. As a result, expression of *marA* is de-repressed, which induces expression of *acrAB* [65].

Other factors involved in regulation of *acrAB-tolC* (post-transcription and post-translation)

The Lon protease is an ATP-dependent protease belonging to the AAA (ATPases associated with a variety of cellular activities) super-family and can be found in both *Salmonella* and *E. coli* [41, 52]. In *E. coli*, Lon has been shown to play a role in regulation of MarA and SoxS at a post-translational level by proteolytic degradation (Fig. 1) [18]. Levy *et al*, demonstrated that mutations in Lon leads to

increased AcrAB efflux and MDR as MarA is not degraded [41]. The impact upon MDR was greatest when combined with a *marR* mutation [41].

Lon performs similar functions within *Salmonella* as *E. coli*, with additional roles in Salmonella Pathogenicity Island (SPI)-1 gene expression and heme biosynthesis [66, 69]. The Lon protease also proteolytically degrades RamA (Fig. 1) [52]. Lon recognises the N-terminal region of RamA, and by binding to this site can degrade the protein [52]. In this way levels of RamA can be re-set to basal levels when the protein is no longer required or there is no longer an inducing stimulus.

Ricci *et al*., [51] recently showed that the global regulator Carbon Storage Regulator A (CsrA) is involved in the regulation of AcrAB (Fig. 1). CsrA is an RNA binding protein that acts as a global regulator of diverse genes. CsrA binds directly to the 5' end of the *acrAB* transcript. This in turn alters RNA secondary structure preventing the formation of a repressive RNA structure that impedes binding of the ribosome, thus allowing for more efficient translation of the AcrAB proteins [51].

There are subtle differences between regulation of AcrAB-TolC in different species of Enterobacteriaceae

A range of local and global regulators with complex interactions tightly regulates the AcrAB-TolC pump. In *E. coli*, MarA, Sox and Rob have such a tightly woven mechanism of interaction that their target site is termed the *mar/sox/rob* regulon. Each regulator responds to environmental signals as well as each other allowing precise control of the AcrAB-TolC efflux pump and other target genes.

In *Salmonella*, RamA is the predominant regulator, but MarA, SoxS and Rob also influence AcrAB-TolC and cause over-expression and MDR. Mutations causing

over expression of SoxS lead to increased resistance to fluoroquinolones, chloramphenicol and tetracycline, but confers less MDR when compared to mutations in *ramA* or *ramR* (Table 1) [75]. Mutations in *rob* induces *mgtA* transcription which is involved in Mg^{2+} transport; this can confer tolerance to cyclohexane [7].

In addition to Mar, Sox, Rob, there are other factors in some other Enterobacteriaceae species not found in *E. coli* or *Salmonella*. For instance, in *Klebsiella pneumoniae* there is *romA* and *rarA*, which are further regulators of the AcrAB pump. RomA is a second transcription factor that can act independently of RamA. It is in the *romA-ramA* locus regulated by RamR [59]. A further factor in *K. pneumoniae*, RarA, can also be induced independently of the other regulators and when over-expressed can also cause increased expression of *acrAB* and MDR [67].

Concluding remarks

RND MDR efflux pumps such as AcrAB-TolC play a vital role in Gram-negative bacteria, and consequently are under complex regulation. This allows for temporary activation of pumps under certain conditions and a rapid return to basal states. This implies that over-expression is not beneficial to the bacterium; possibly because of high-energy requirements. Constitutive de-repression conferring MDR to clinically useful drugs is due to evolutionary pressure of drug exposure. In order to fully understand the mechanisms involved it is essential that the full repertoire of regulatory factors, including those that are species specific, are identified. This knowledge will help identify those factors that could be targets for drug discovery and which could be inhibited as a mechanism to down-regulate MDR efflux and sensitise bacteria to antibiotics susceptible to efflux.

References

[1] Abouzeed YM, Baucheron S, Cloeckaert A. *ramR* mutations involved in efflux-mediated multidrug resistance in *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemother 2008;52:2428-34.
[2] Alekshun MN, Levy SB. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob Agents Chemother 1997;41:2067-75.
[3] Alekshun MN, Levy SB. Alteration of the repressor activity of MarR, the negative regulator of the *Escherichia coli marRAB* locus, by multiple chemicals *in vitro*. J Bacteriol 1999;181:4669-72.
[4] Alekshun MN, Levy SB. The *mar* regulon: multiple resistance to antibiotics and other toxic chemicals. Trends Microbiol 1999;7:410-3.
[5] Anes J, McCusker MP, Fanning S, Martins M. The ins and outs of RND efflux pumps in *Escherichia coli*. Front Microbiol 2015;6:587.
[6] Bailey AM, Paulsen IT, Piddock LJ. RamA confers multidrug resistance in *Salmonella enterica* via increased expression of *acrB*, which is inhibited by chlorpromazine. Antimicrob Agents Chemother 2008;52:3604-11.
[7] Barchiesi J, Castelli ME, Soncini FC, Vescovi EG. *mgtA* Expression is induced by *rob* overexpression and mediates a *Salmonella enterica* resistance phenotype. J Bacteriol 2008;190:4951-8.
[8] Baucheron S, Coste F, Canepa S, Maurel MC, Giraud E, Culard F, et al. Binding of the RamR repressor to wild-type and mutated promoters of the RamA gene involved in efflux-mediated multidrug resistance in *Salmonella enterica* serovar Typhimurium. Antimicrob Agents Chemother 2012;56:942-8.
[9] Baucheron S, Nishino K, Monchaux I, Canepa S, Maurel MC, Coste F, et al. Bile-mediated activation of the *acrAB* and *tolC* multidrug efflux genes occurs mainly through transcriptional derepression of *ramA* in *Salmonella enterica* serovar Typhimurium. J Antimicrob Chemother 2014;69:2400-6.
[10] Blair JM, Richmond GE, Piddock LJ. Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. Future Microbiol 2014;9:1165-77.
[11] Chubiz LM, Glekas GD, Rao CV. Transcriptional cross talk within the *mar-sox-rob* regulon in *Escherichia coli* is limited to the *rob* and *marRAB* operons. J Bacteriol 2012;194:4867-75.
[12] Cohen SP, Hachler H, Levy SB. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J Bacteriol 1993;175:1484-92.
[13] Cohen SP, Levy SB, Foulds J, Rosner JL. Salicylate induction of antibiotic resistance in *Escherichia coli:* activation of the *mar* operon and a *mar*-independent pathway. J Bacteriol 1993;175:7856-62.
[14] Egan SM. Growing repertoire of AraC/XylS activators. J Bacteriol 2002;184:5529-32.
[15] Everett MJ, Jin YF, Ricci V, Piddock LJ. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. Antimicrob Agents Chemother 1996;40:2380-6.
[16] Gillette WK, Martin RG, Rosner JL. Probing the *Escherichia coli* transcriptional activator MarA using alanine-scanning mutagenesis: residues important for DNA binding and activation. J Mol Biol 2000;299:1245-55.
[17] Griffith KL, Fitzpatrick MM, Keen EF, 3rd, Wolf RE, Jr. Two functions of the C-terminal domain of *Escherichia coli* Rob: mediating "sequestration-dispersal" as a novel off-on switch for regulating Rob's activity as a transcription activator and preventing degradation of Rob by Lon protease. J Mol Biol 2009;388:415-30.

[18] Griffith KL, Shah IM, Wolf RE, Jr. Proteolytic degradation of *Escherichia coli* transcription activators SoxS and MarA as the mechanism for reversing the induction of the superoxide (SoxRS) and multiple antibiotic resistance (Mar) regulons. Mol Microbiol 2004;51:1801-16.
[19] Hagman KE, Lucas CE, Balthazar JT, Snyder L, Nilles M, Judd RC, et al. The MtrD protein of *Neisseria gonorrhoeae* is a member of the resistance/nodulation/division protein family constituting part of an efflux system. Microbiology 1997;143 (Pt 7):2117-25.
[20] Hidalgo E, Leautaud V, Demple B. The redox-regulated SoxR protein acts from a single DNA site as a repressor and an allosteric activator. EMBO J 1998;17:2629-36.
[21] Hirakawa H, Takumi-Kobayashi A, Theisen U, Hirata T, Nishino K, Yamaguchi A. AcrS/EnvR represses expression of the *acrAB* multidrug efflux genes in *Escherichia coli*. J Bacteriol 2008;190:6276-9.
[22] Jair KW, Fawcett WP, Fujita N, Ishihama A, Wolf RE, Jr. Ambidextrous transcriptional activation by SoxS: requirement for the C-terminal domain of the RNA polymerase alpha subunit in a subset of *Escherichia coli* superoxide-inducible genes. Mol Microbiol 1996;19:307-17.
[23] Kwon HJ, Bennik MH, Demple B, Ellenberger T. Crystal structure of the *Escherichia coli* Rob transcription factor in complex with DNA. Nat Struct Biol 2000;7:424-30.
[24] Lawler AJ, Ricci V, Busby SJ, Piddock LJ. Genetic inactivation of *acrAB* or inhibition of efflux induces expression of *ramA*. J Antimicrob Chemother 2013;68:1551-7.
[25] Li XZ, Livermore DM, Nikaido H. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob Agents Chemother 1994;38:1732-41.
[26] Li XZ, Ma D, Livermore DM, Nikaido H. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa:* active efflux as a contributing factor to beta-lactam resistance. Antimicrob Agents Chemother 1994;38:1742-52.
[27] Li XZ, Plesiat P, Nikaido H. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev 2015;28:337-418.
[28] Lin J, Michel LO, Zhang Q. CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. Antimicrob Agents Chemother 2002;46:2124-31.
[29] Ma D, Alberti M, Lynch C, Nikaido H, Hearst JE. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol Microbiol 1996;19:101-12.
[30] Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. Antimicrob Agents Chemother 2001;45:3375-80.
[31] Martin RG, Gillette WK, Martin NI, Rosner JL. Complex formation between activator and RNA polymerase as the basis for transcriptional activation by MarA and SoxS in *Escherichia coli*. Mol Microbiol 2002;43:355-70.
[32] Martin RG, Gillette WK, Rhee S, Rosner JL. Structural requirements for marbox function in transcriptional activation of *mar/sox/rob* regulon promoters in *Escherichia coli:* sequence, orientation and spatial relationship to the core promoter. Mol Microbiol 1999;34:431-41.
[33] Martin RG, Jair KW, Wolf RE, Jr., Rosner JL. Autoactivation of the *marRAB* multiple antibiotic resistance operon by the MarA transcriptional activator in *Escherichia coli*. J Bacteriol 1996;178:2216-23.

[34] Martin RG, Rosner JL. Fis, an accessorial factor for transcriptional activation of the *mar* (multiple antibiotic resistance) promoter of *Escherichia coli* in the presence of the activator MarA, SoxS, or Rob. J Bacteriol 1997;179:7410-9.
[35] Martin RG, Rosner JL. The AraC transcriptional activators. Curr Opin Microbiol 2001;4:132-7.
[36] Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2000;44:3322-7.
[37] McDermott PF, McMurry LM, Podglajen I, Dzink-Fox JL, Schneiders T, Draper MP, et al. The *marC* gene of *Escherichia coli* is not involved in multiple antibiotic resistance. Antimicrob Agents Chemother 2008;52:382-3.
[38] Michan C, Manchado M, Pueyo C. SoxRS down-regulation of *rob* transcription. J Bacteriol 2002;184:4733-8.
[39] Miller PF, Gambino LF, Sulavik MC, Gracheck SJ. Genetic relationship between *soxRS* and *mar* loci in promoting multiple antibiotic resistance in *Escherichia coli*. Antimicrob Agents Chemother 1994;38:1773-9.
[40] Nakajima H, Kobayashi K, Kobayashi M, Asako H, Aono R. Overexpression of the *robA* gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. Appl Environ Microbiol 1995;61:2302-7.
[41] Nicoloff H, Perreten V, McMurry LM, Levy SB. Role for tandem duplication and lon protease in AcrAB-TolC- dependent multiple antibiotic resistance (Mar) in an *Escherichia coli* mutant without mutations in *marRAB* or *acrRAB*. J Bacteriol 2006;188:4413-23.
[42] Nikaido E, Yamaguchi A, Nishino K. AcrAB multidrug efflux pump regulation in *Salmonella enterica* serovar Typhimurium by RamA in response to environmental signals. J Biol Chem 2008;283:24245-53.
[43] Nishino K, Hayashi-Nishino M, Yamaguchi A. H-NS modulates multidrug resistance of *Salmonella enterica* serovar Typhimurium by repressing multidrug efflux genes *acrEF*. Antimicrob Agents Chemother 2009;53:3541-3.
[44] Nishino K, Yamaguchi A. Role of histone-like protein H-NS in multidrug resistance of *Escherichia coli*. J Bacteriol 2004;186:1423-9.
[45] Nunoshiba T, Hidalgo E, Li Z, Demple B. Negative autoregulation by the *Escherichia coli* SoxS protein: a dampening mechanism for the *soxRS* redox stress response. J Bacteriol 1993;175:7492-4.
[46] Okusu H, Ma D, Nikaido H. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J Bacteriol 1996;178:306-8.
[47] Olliver A, Valle M, Chaslus-Dancla E, Cloeckaert A. Role of an *acrR* mutation in multidrug resistance of *in vitro*-selected fluoroquinolone-resistant mutants of *Salmonella enterica* serovar Typhimurium. FEMS Microbiol Lett 2004;238:267-72.
[48] Pumbwe L, Piddock LJ. Identification and molecular characterisation of CmeB, a *Campylobacter jejuni* multidrug efflux pump. FEMS Microbiol Lett 2002;206:185-9.
[49] Rahmati S, Yang S, Davidson AL, Zechiedrich EL. Control of the *AcrAB* multidrug efflux pump by quorum-sensing regulator SdiA. Mol Microbiol 2002;43:677-85.
[50] Rhee S, Martin RG, Rosner JL, Davies DR. A novel DNA-binding motif in MarA: the first structure for an AraC family transcriptional activator. Proc Natl Acad Sci U S A 1998;95:10413-8.

[51] Ricci V, Attah V, Overton T, Grainger DC, Piddock LJV. CsrA maximizes expression of the AcrAB multidrug resistance transporter. Nucleic Acids Research 2017;In Press.
[52] Ricci V, Blair JM, Piddock LJ. RamA, which controls expression of the MDR efflux pump AcrAB-TolC, is regulated by the Lon protease. J Antimicrob Chemother 2014;69:643-50.
[53] Ricci V, Busby SJ, Piddock LJ. Regulation of RamA by RamR in *Salmonella enterica* serovar Typhimurium: isolation of a RamR superrepressor. Antimicrob Agents Chemother 2012;56:6037-40.
[54] Ricci V, Loman N, Pallen M, Ivens A, Fookes M, Langridge GC, et al. The TCA cycle is not required for selection or survival of multidrug-resistant *Salmonella*. J Antimicrob Chemother 2012;67:589-99.
[55] Ricci V, Piddock LJ. Ciprofloxacin selects for multidrug resistance in *Salmonella enterica* serovar Typhimurium mediated by at least two different pathways. J Antimicrob Chemother 2009;63:909-16.
[56] Ricci V, Piddock LJ. Only for substrate antibiotics are a functional AcrAB-TolC efflux pump and RamA required to select multidrug-resistant *Salmonella* Typhimurium. J Antimicrob Chemother 2009;64:654-7.
[57] Ricci V, Tzakas P, Buckley A, Piddock LJ. Ciprofloxacin-resistant *Salmonella enterica* serovar Typhimurium strains are difficult to select in the absence of AcrB and TolC. Antimicrob Agents Chemother 2006;50:38-42.
[58] Rosenberg EY, Bertenthal D, Nilles ML, Bertrand KP, Nikaido H. Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. Mol Microbiol 2003;48:1609-19.
[59] Rosenblum R, Khan E, Gonzalez G, Hasan R, Schneiders T. Genetic regulation of the *ramA* locus and its expression in clinical isolates of *Klebsiella pneumoniae*. Int J Antimicrob Agents 2011;38:39-45.
[60] Rosner JL, Dangi B, Gronenborn AM, Martin RG. Posttranscriptional activation of the transcriptional activator Rob by dipyridyl in *Escherichia coli*. J Bacteriol 2002;184:1407-16.
[61] Schneiders T, Levy SB. MarA-mediated transcriptional repression of the *rob* promoter. J Biol Chem 2006;281:10049-55.
[62] Schumacher MA, Brennan RG. Structural mechanisms of multidrug recognition and regulation by bacterial multidrug transcription factors. Mol Microbiol 2002;45:885-93.
[63] Sharma P, Haycocks JRJ, Middlemiss AD, Kettles R, Sellars LE, Ricci V, et al. The multiple antibiotic resistance operon of enteric bacteria controls DNA repair and outer membrane integrity. Nat Commun 2017;In Press.
[64] Su CC, Rutherford DJ, Yu EW. Characterization of the multidrug efflux regulator AcrR from *Escherichia coli*. Biochem Biophys Res Commun 2007;361:85-90.
[65] Sulavik MC, Gambino LF, Miller PF. The MarR repressor of the multiple antibiotic resistance (*mar*) operon in *Escherichia coli:* prototypic member of a family of bacterial regulatory proteins involved in sensing phenolic compounds. Mol Med 1995;1:436-46.
[66] Takaya A, Kubota Y, Isogai E, Yamamoto T. Degradation of the HilC and HilD regulator proteins by ATP-dependent Lon protease leads to downregulation of *Salmonella* pathogenicity island 1 gene expression. Mol Microbiol 2005;55:839-52.
[67] Veleba M, Higgins PG, Gonzalez G, Seifert H, Schneiders T. Characterization of RarA, a novel AraC family multidrug resistance regulator in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2012;56:4450-8.

[68] Vinue L, McMurry LM, Levy SB. The 216-bp *marB* gene of the *marRAB* operon in *Escherichia coli* encodes a periplasmic protein which reduces the transcription rate of *marA*. FEMS Microbiol Lett 2013;345:49-55.
[69] Wang L, Wilson S, Elliott T. A mutant HemA protein with positive charge close to the N terminus is stabilized against heme-regulated proteolysis in *Salmonella typhimurium*. J Bacteriol 1999;181:6033-41.
[70] Warner DM, Levy SB. Different effects of transcriptional regulators MarA, SoxS and Rob on susceptibility of *Escherichia coli* to cationic antimicrobial peptides (CAMPs): Rob-dependent CAMP induction of the *marRAB* operon. Microbiology 2010;156:570-8.
[71] Webber MA, Piddock LJ. Absence of mutations in *marRAB* or *soxRS* in *acrB*-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of *Escherichia coli*. Antimicrob Agents Chemother 2001;45:1550-2.
[72] Webber MA, Talukder A, Piddock LJ. Contribution of mutation at amino acid 45 of AcrR to *acrB* expression and ciprofloxacin resistance in clinical and veterinary *Escherichia coli* isolates. Antimicrob Agents Chemother 2005;49:4390-2.
[73] White DG, Goldman JD, Demple B, Levy SB. Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA, soxS*, or *robA* in *Escherichia coli*. J Bacteriol 1997;179:6122-6.
[74] Yamasaki S, Nikaido E, Nakashima R, Sakurai K, Fujiwara D, Fujii I, et al. The crystal structure of multidrug-resistance regulator RamR with multiple drugs. Nat Commun 2013;4:2078.
[75] Zheng J, Cui S, Meng J. Effect of transcriptional activators RamA and SoxS on expression of multidrug efflux pumps AcrAB and AcrEF in fluoroquinolone-resistant *Salmonella* Typhimurium. J Antimicrob Chemother 2009;63:95-102.

Legend to Figures

Figure 1. Transcriptional regulation of the *acrAB* (red and blue) and *tolC* (yellow) regulon by *E. coli marRAB* (green) and *S. enterica ramRA* (purple) genes. Post transcriptional regulation of *acrAB* by CsrA (grey) and post translational regulation of RamA by Lon protease (orange).

Figure 2: Structure and binding site of MarA: A) MarA (purple) binds the major grooves of DNA (black) by its DNA binding domain (generated via Rasmol by using PDB 1B10). B) The 20 bp consensus sequence bound by MarA, which is highly degenerate and asymmetric. Adapted from Rhee et al.,1998[24]. Similar binding may be observed in the case where RamA binds DNA.